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16. ABSTRACT

The discovery that phorbol ester tumor promoters inhibit metabolic cooperation between cultured cells in proportion to their promoting activity in vivo suggests that such inhibition may be a mechanism in tumor promotion. Because metabolic cooperation appears to be essential for normal cell differentiation and tissue development, the concept that chemicals might be toxic by blocking this process may, in theory, be extended to reproductive system toxicants. We are testing these hypotheses using an assay developed with V79 cells. Effects of chemicals on metabolic cooperation are assessed by adding test chemical and selective agent (6-thioguanine) simultaneously to co-cultivated mutant ($HGPRT^-$) and wild-type ($HGPRT^+$) cells in reconstructed mutant selection experiments. Effects of test chemicals are measured as a function of mutant cell recovery relative to that in a control. Our studies indicate that in addition to phorbol ester promoters (PMA, PDBu), nonphorbol ester promoters (BHT, cyclamate), cocarcinogenic or teratogenic solvents (ETOH, DMSO), and potentially epigenetic carcinogens (DEPH, NTA) inhibit metabolic cooperation. Some promoters (phenol) do not appear to block metabolic cooperation directly, but yield metabolites which do. In Press.

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EFFECTS OF STRUCTURALLY DIVERSE CHEMICALS ON METABOLIC COOPERATION IN VITRO

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INTRODUCTION

Permeable intercellular (gap) junctions allowing the direct exchange of small ions and molecules between cells in coupled populations are a common feature of most animal tissues (24,26,35,62,73). Research suggests that such gap-junctional communication, defined as metabolic cooperation (46,91,92), is necessary in plants and animals for coordination and control of cell proliferation, differentiation, and other activities which collectively result in the integration of individual cells into organized tissues (73). Specifically, metabolic cooperation is implicated in developmental signalling, embryonic induction, growth control, cell nutrition, the control of hormonal responses, and general tissue homeostasis (41,62,72,80,114), thus suggesting the fundamental importance of this biological process to developing and adult multicellular organisms.

The recent discovery (67,116) that phorbol ester tumor promoters inhibit metabolic cooperation between several types of cultured cells suggests that inhibited metabolic cooperation is a mechanism for tumor promotion. Because metabolic cooperation appears to be important for normal embryonic development, the concept has been extended to include some reproductive system effects (99), including teratogenesis (98). These ideas form a general hypothesis which predicts that inhibition of metabolic cooperation in adults may result in tumor promotion,

whereas its inhibition in developing embryos may induce teratogenesis.

The hypothesis that tumors may be promoted through inhibition of metabolic cooperation assumes that the carcinogenic process is multi-step and that the growth of premalignant (initiated) cells may be regulated via signals from communicating normal cells. Multiple stage carcinogenesis is now well documented (16,38,44,47,68,70,109,113) for many organs in several animal models, and is extensively reviewed (14,15,27,79,85,87). Regulation of initiated cell growth through metabolic cooperation with normal cells awaits demonstration, but evidence suggesting a role for metabolic cooperation in the regulation of cell growth is abundant. For example, cell strains defective for both growth control (density-dependent inhibition of replication) and metabolic cooperation have been isolated and characterized (60,61). The segregation patterns of these and several other biochemical traits resulting from hybridization experiments with normal and defective cells suggest that metabolic cooperation and loss of growth control are genetically linked (2,4,12). Cells defective for both traits are also tumorigenic in appropriate animal hosts (12,34,60).

Potter views carcinogenesis as a fundamental problem of local intercellular communication (74,75) and uses the concept to propose that certain tissue-specific inhibitors of cell proliferation (chalones or chalone-like substances) might control cell growth through regulation of metabolic cooperation. Closely associated with this concept is the likely role of cyclic AMP (cAMP) and the phosphorylation of certain proteins by the kinases it activates (76). Indeed, Wiener and Loewenstein recently report (107) that mutant Chinese hamster ovarian cells deficient for a protein kinase (cAMP receptor) are

also deficient for gap-junctional communication, and that the latter deficiency is restored when the missing protein kinase is supplied exogenously.

Additional evidence suggests a connection between metabolic cooperation and cell growth regulation. Recently demonstrated (59) is the transfer of antiproliferative activity from interferron-induced cells to recipient cells co-cultivated in vitro. Transfer occurred only when donor and recipient cells were in physical contact, further implicating metabolic cooperation in the regulation of cell growth. In addition, Friedman and Steinberg report (37) that early and late-stage, premalignant human colon cells communicate effectively through metabolic cooperation whereas cells from an established human colon carcinoma do not. Moreover, the tumor promoter, phorbol-12-myristate-13-acetate (PMA), inhibits metabolic cooperation between the late-stage but not early-stage cells, suggesting that promoters may facilitate the transition of some premalignant cells to malignancy by blocking metabolic cooperation. These results support recent observations that many tumor promoters and several carcinogens inhibit metabolic cooperation in vitro (42,63,97,110).

We are testing the general hypothesis through correlation studies with selected tumor promoters, carcinogens, and other chemicals, some of which are also teratogenic. In this paper, we summarize results for eighteen chemicals tested in our laboratory and discuss the results in relation to general hypothesis predictions.

METABOLIC COOPERATION ASSAY

Gap junctions form between many types of cultured animal cells and metabolic cooperation between them may be measured by several techniques (60,92). We use the selection phase of the V79/HGPRT mutation assay (47) modified to facilitate detection of chemicals which inhibit metabolic cooperation. This approach, described by Yotti, et al. (18), and Trosko, et al. (95), involves the co-cultivation of mutant ($HGPRT^-$) and wild-type ($HGPRT^+$) V79 Chinese hamster lung fibroblasts. The $HGPRT^-$ cells are deficient for the enzyme hypoxanthine-guanine phosphoribosyl transferase (HGPRTase) and are assumed to possess mutations in the structural gene coding for the enzyme (21). HGPRTase is a purine salvage enzyme which catalyses the conversion of preformed purine bases (hypoxanthine and guanine) into their respective nucleotides (21) which are then incorporated into nucleic acid. In addition to these normal purine bases, HGPRTase also catalyses the conversion of certain abnormal bases, such as 8-azaguanine and 6-thioguanine, into their respective nucleotides. Because the cytoplasmic membrane is not permeable to nucleotides, such metabolites tend to be incorporated into nucleic acid in those cells within which they arise (45,58). Upon incorporation, the abnormal nucleotides are highly toxic. Consequently, 6TG is toxic to $HGPRT^+$ but not $HGPRT^-$ cells. This is the principle underlying $HGPRT^-$ cell selection by 6TG from mixed populations in mutation experiments. In such experiments, 6TG is added to the growth medium to eliminated $HGPRT^+$ cells. $HGPRT^-$ cells survive and grow into macroscopic colonies, providing they do not establish physical contact with $HGPRT^+$ cells during the selective process.

If contact occurs, gap junctions will form and the toxic metabolite of 6TG is transferred from $HGPRT^+$ to $HGPRT^-$ cells, resulting in mutant cell loss. In mutation experiments, the probability of $HGPRT^-$ cell loss is minimized by screening mixed populations at low densities where the probability of cell contact is similarly low. The loss of $HGPRT^-$ cells through metabolic cooperation with $HGPRT^+$ cells, while undesirable in mutation experiments, provides a convenient way to identify chemicals which inhibit metabolic cooperation. The assay for metabolic cooperation becomes a reconstructed selection experiment in which known numbers of $HGPRT^-$ cells are co-cultivated with enough $HGPRT^+$ cells to ensure $HGPRT^-$ cell loss unless 'rescued' by test chemicals which inhibit metabolic cooperation. The effects of test chemicals (added simultaneously with 6TG) are measured as a function of $HGPRT^-$ cell recovery relative to the usual low recovery of these cells in solvent controls. Currently, no exogenous metabolizing system is used. Thus, because V79 cells have a limited capacity to metabolize chemicals (18), observed responses are assumed to result from the efffects of unmetabolized test substances. The present testing protocol and statistical approaches for data analysis are published (95). For results described below, the effects of chemicals on metabolic cooperation were assessed at doses permitting at least 70 percent survival in low-density, toxicity tests performed with $HGPRT^-$ cells only.

RESULTS AND DISCUSSION

Test results are summarized in Table 1. Chemicals had either no effect on metabolic cooperation, inhibited metabolic cooperation, or enhanced it. Chemicals were judged to have no effect on metabolic cooperation if mutant recovery at all doses assessed was not different from that in the solvent control. Inhibition of metabolic cooperation resulted in an increase in mutant cell recovery over background, whereas enhancement was characterized by a decrease in mutant recovery relative to background.

Tumor Promoters

Five tumor promoters of varying tissue specificity and efficacy were tested. PMA, a potent promoter of mouse skin tumors (43,101), increased mutant recovery at 0.1 ng/ml and induced complete mutant recovery at 1 ng/ml (63). Phorbol-12,13-dibutyrate (PDBu), a much weaker promoter of similar tumors (87), increased mutant recovery to 60 percent at 10 ng/ml. In these experiments, background recovery averaged about 10 percent at 10 ng/ml. PMA and PDBu thus inhibited metabolic cooperation in proportion to their promoting activity in vivo. Similar findings are reported (116) by others. Inhibition of metabolic cooperation at the ng/ml level suggests that PMA and PDBu induce effects through interaction with structurally specific receptors (55). Binding studies with specific phorbol esters provide convincing evidence for such receptors(10,107).

Butylated hydroxytoluene (BHT), a promoter of liver tumors in rats pretreated with 2-acetylaminofluorene (71), and lung tumors in mice pretreated with urethane (113), inhibited metabolic cooperation at nontoxic concentrations (25 µg/ml or less) in two experiments,

TABLE 1. Effects of Structurally Diverse Chemicals on Metabolic Cooperation Between Cultured V79 Chinese Hamster Lung Fibroblasts

No Effect	Inhibition	Enhancement
3,4-benzopyrene	phorbol-12-myristate-13-acetate	
	phorbol-12,13-dibutyrate	
	butylated hydroxytoluene	
	sodium cyclamate	
phenol	catechol	2-methoxyphenol
phenylglucuronide	quinol	
	hydroxyquinol	
	1,4-benzoquinone	
	phenysulfate	
	di-(2-ethylhexyl) phthalate	
	trisodium nitrilotriacetate	
	4-chlorobiphenyl	
	ethanol dimethylsulfoxide	

but had no effect on metabolic cooperation at these same concentrations in two other experiments. Because BHT showed a dose response in those experiments where it inhibited metabolic cooperation, and because BHT is reported (97) by others to inhibit metabolic cooperation in this assay, we classified BHT as an inhibitor of metabolic cooperation. It is possible that factors influencing the effect of BHT on metabolic cooperation were not controlled in our experiments. BHT is considered to be an atypical promoter in that it appears to promote tumorigenesis in vivo only if carcinogenic doses of initiator are given (113). BHT promotes the in vitro transformation of C3H/10T1/2 cells pretreated with subtransforming doses of 20-methylcholanthrene (29). The teratogenicity of BHT is controversial (5).

At high doses, sodium cyclamate (SCM) promotes bladder tumors in rats pretreated with nitrosomethylurea (44). SCM inhibited metabolic cooperation in a dose-dependent manner, also at high (1-4 mg/ml) concentrations. Saccharin, a similarly weak promoter (44), requires similar concentrations to inhibit metabolic cooperation in V79 cells to the same extent (94). SCM reversibly inhibits electrical coupling (produced by the transfer of ions through metabolic cooperation) between human epithelial cells in culture (33). There is no conclusive evidence that SCM is teratogenic in animals, although there is circumstantial evidence for this in humans (49).

Phenol, a weak promoter of mouse skin tumors (13,103), had no effect on metabolic cooperation at all concentrations (400 μg/ml or less) tested. Concentrations greater than 300 μg/ml were toxic. This result suggested that phenol did not fit the hypothesis that tumor promoters inhibit metabolic cooperation. Because the skin is

important as a drug-metabolizing organ (69), seven metabolites of phenol (111) were tested on the hypothesis that promotion by some chemicals might be mediated through metabolic products. Four minor metabolies (1,4-benzoquinone, catechol, hydroxyquinol, and quinol) weakly inhibited metabolic cooperation in a dose-related fashion at moderate (1-3 μg/ml) concentrations (63). Phenylsulfate, a major conjugation product, also weakly inhibited metabolic cooperation, but at high (1-4 mg/ml) concentrations and without dose response. Phenylglucuronide, another major conjugation product, had no effect on metabolic cooperation and was nontoxic at all concentrations (7.5 mg/ml or less) tested. A methylated derivative of catechol, 2-methyoxyphenol, enhanced metabolic cooperation in a dose-dependent manner between 20 and 70 μg/ml. Inhibition of metabolic cooperation by five metabolites of phenol indicates that phenol is not an exception to the hypothesis. Moreover, this result indicates the need to consider metabolites when testing the hypothesis. Recognition of the need to consider metabolites is especially important in view of the fact that some workers have reported (22,30,54) that inhibition of metabolic cooperation is not a property common to all tumor promoters. Some of these promoters inhibit metabolic cooperation when tested under conditions more suitable for detection of weaker substances (95). In other cases (30), effects may be mediated through metabolites.

The weakness of phenol's metabolites in inhibiting metabolic cooperation correlates with the weakness of phenol as a promoter in vivo (13). The pharmacological significance of enhanced metabolic cooperation requires investigation, particularly with respect to

antipromotion effects. For example, retinoic acid, an antipromoter under some conditions (103), increases the number of gap junctions on the membranes of some cells (37,57), thereby increasing the opportunity for metabolic cooperation. Chemicals causing cells to increase in size may also enhance metabolic cooperation by increasing the probability of cell contact (6). Alternatively, chemicals causing cells to decrease in size or to round up from cytotoxic effects, may inhibit metabolic cooperation through physical disruption of gap junctions (30). Chemicals may also effect metabolic cooperation through effects on the size and composition of intracellular pools of transferable substances (7,45). Alternative explanations (e.g., cytotoxicity) for the promoting activity of phenol cannot be ruled out, particularly as catechol and quinol are reported (13,103) to be inactive as mouse skin tumor promoters. These substances may, however, be promoters for other organs. Additional in vivo studies with catechol and quinol may be required. Phenol is not teratogenic in rats according to one study (66), but induces developmental defects in submammalian species, (8,9). Interestingly, phenol shows activity without exogenous metabolism in a recently-developed, in vitro test for teratogens (19).

Selected Carcinogens

3,4-Benzopyrene (BP), a complete carcinogen which may promote (102) as well as initiate (103), had no effect on metabolic cooperation at nontoxic (3 μg/ml or less) concentrations (63). As BP is extensively metabolized and requires metabolism to a carcinogenic form (39), it is possible that promoting effects are mediated through metabolies (65). Specific metabolites should therefore be evaluated for their

effects on metabolic cooperation. BP, however, fails to inhibit metabolic cooperation between co-cultivated HGPRTase-deficient ARL14 cells and freshly-isolated rat hepatocytes (93) which should be capable of metabolizing BP. Perhaps, in the cell culture system, not enough metabolite is produced to inhibit metabolic cooperation. BP is also teratogenic (82).

Two carcinogenic but apparently nonmutagenic chemicals were tested. The trisodium salt of nitrilotriacetic acid (NTA), a carcinogen at high doses in rats (20,40), inhibited metabolic cooperation in a dose-related manner at high (1-3 mg/ml) concentrations (63). NTA may inhibit metabolic cooperation by chelating calcium, an important element in the regulation of metabolic cooperation (62). Evidence from animals studies (1) indicates that the toxic effects of NTA correlate with the extraction of calcium via chelation from tissue intercellular spaces. With the exception of clastogenic effects at high doses in somatic cells (11,53), NTA is not genotoxic in a variety of assays (31,34,56). NTA does not appear to be teratogenic in mammals (112), but developmental defects are reported (8,9) for nonmammalian species.

Di-(2-ethylhexyl) phthalate (DEHP) presents a similar situation. DEHP is carcinogenic at high doses in F344 rats and B6C3F1 mice, but, with the possible exception of chromosomal effects, is not genotoxic (50). DEHP inhibited metabolic cooperation in a dose-dependent fashion at moderate (1-30 μg/ml) concentrations (63). DEHP promotes JB-6 epidermal cells to anchorage independence and tumorigenicity (28), and is a potent promoter of hepatic tumors in B6C3F1 mice (105). DEHP is also teratogenic at high doses in rats and mice (81).

As a class, polychlorinated biphenyls (PCBs) induce a broad range of biological effects, including carcinogenesis (48), teratogenesis (106), and tumor promotion (51,77), but not genotoxicity (78). In addition, some are strong inhibitors of metabolic cooperation (48,100). The monochlorinated biphenyl, 4-chlorobiphenyl (4-CBP), was tested for comparative purposes. 4-CBP inhibited metabolic cooperation in a dose-dependent manner at moderate (3-20 μg/ml) concentrations (63). Concentrations greater than 20 μg/ml were toxic. Inhibition of metabolic cooperation by 4-CBP indicates that monochlorinated biphenyls may have tumor-promoting or teratogenic potential.

Solvents

Ethanol (ETOH) and dimethylsulfoxide (DMSO) were evaluated for their effects on metabolic cooperation as they are frequently used to prepare chemicals for testing. ETOH had no effect on metabolic cooperation at concentrations of 5 mg/ml or less (63), but inhibited metabolic cooperation in a dose-dependent fashion at high (10-20 mg/ml) concentrations. Concentrations greater than 20 mg/ml were toxic. ETOH is considered to be cocarcinogenic in humans (114), is perhaps teratogenic in humans (88), and is teratogenic in rats and mice (23, 64). In addition, ETOH is a surface-active agent and is predicted to have tumor promoting properties for this reason (17).

DMSO had no effect on metabolic cooperation at concentrations of 2.5 mg/ml or less (63), but inhibited metabolic cooperation in a dose-related manner between 5 and 25 mg/ml. A similar finding for DMSO is reported by Dorman and Boreiko (30). DMSO is teratogenic in mice (52) and hamsters (36), but apparently not rats (52, 89), suggesting species differences. DMSO is not carcinogenic for mouse skin according

to one study (90), and, in fact, strongly inhibits the promoting activity of PMA on mouse skin (87). In view of the rapid and complete absorption of DMSO through the intact dermis and other membranes (25), inhibition of PMA-induced promotion by DMSO may reflect the rapid absorption of dissolved PMA beyond the zone of initiated cells. Moreover, the fact that DMSO inhibits promotion by PMA infers little about the promoting potential of DMSO, as other promoters (e.g., BHT and acetic acid (86)) also inhibit promotion by PMA (87). We are unaware of any studies in which the promoting potential of DMSO has been evaluated.

SUMMARY

The effects on metabolic cooperation of eighteen chemicals, summarized and discussed in this paper, largely support the general hypothesis that tumor promoters and some teratogens inhibit metabolic cooperation. Consequently, in vitro assays designed to assess the effects of chemicals on metabolic cooperation may be predictive of these events. If so, such assays may be useful as screening tests to identify chemicals requiring risk evaluation.

The inhibiting effects on metabolic cooperation by metabolites of phenol, but not phenol, indicates the importance of considering metabolites when testing the hypothesis. The discovery that chemicals enhance was well as inhibit metabolic cooperation requires pharmacological evaluation and suggests the need to understand how chemicals influencing metabolic cooperation differently might interact to produce an effect. Also needed are more specific predictions of those reproductive system effects likely to arise through disturbances of metabolic cooperation so that this aspect of the general hypothesis can be adequately tested. Additional studies are required to better establish the role of metabolic cooperation in biological systems and the biological consequences of effects on this process. In our judgment, results from correlation studies presently support the general hypothesis sufficiently well to justify further investigation into the role of metabolic cooperation as a possible mechanism underlying some apparently different but perhaps related toxicological events.

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